

Rapid ventricular pacing-induced postconditioning attenuates reperfusion injury: effects on peroxynitrite, RISK and SAFE pathways

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Abstract

Background and purpose: Rapid ventricular pacing (RVP) applied before an index ischaemia has anti-ischaemic effects. Here we investigated whether RVP applied after index ischaemia attenuates reperfusion injury and whether peroxynitrite, RISK and SAFE pathways as well as HO-1 are involved in the mechanism of RVP-induced postconditioning.

Experimental approach: Langendorff perfused rat hearts were subjected to 30 min regional ischaemia and 120 min reperfusion with or without ischaemic postconditioning (6x10/10-s reperfusion/ischaemia; IPost) or RVP (6x10/10-s non-pacing/rapid pacing at 600 bpm) applied at the onset of reperfusion.

Key results: Meta-analysis of our previous studies revealed an association of longer reperfusion-induced ventricular tachycardia/fibrillation with decreased infarct size. In the present experiments testing if RVP is cardioprotective, we found that both IPost and RVP significantly decreased infarct size ($38 \pm 5\%$ and $27 \pm 5\%$ vs. $53 \pm 4\%$, $p < 0.05$), however, only RVP attenuated the incidence of reperfusion-induced ventricular tachycardia. Both postconditioning methods increased formation of cardiac 3-nitrotyrosine and superoxide, and non-significantly enhanced Akt phosphorylation at the beginning of reperfusion without affecting Erk1/2 and Stat3, while solely IPost induced HO-1. Application of brief ischaemia/reperfusion cycles or RVP without preceding index ischaemia also facilitated peroxynitrite formation, nevertheless, only brief RVP increased Stat3 phosphorylation.

Conclusions and implications: Application of short periods of RVP at the onset of reperfusion is cardioprotective and increases peroxynitrite formation similarly to IPost, and thus may serve as an alternative postconditioning method. However, downstream mechanisms of the protection elicited by IPost and RVP seem to be partially different.

Keywords: cardioprotection, conditioning, oxidative and nitrative stress, ONOO^- , protein kinase, MAPK, haem oxygenase

1 Abbreviations:

- 2 ANOVA (analysis of variance)
- 3 BSA (bovine serum albumin)
- 4 GAPDH (glyceraldehyde 3-phosphate dehydrogenase)
- 5 ECG (electrocardiogram)
- 6 HO-1 (haem oxygenase 1)
- 7 I/R (ischaemia/reperfusion)
- 8 IPost (ischaemic postconditioning)
- 9 LAD (left anterior descending coronary artery)
- 10 LDH (lactate dehydrogenase)
- 11 RIPA (radioimmunoprecipitation)
- 12 RISK (reperfusion injury salvage kinase)
- 13 SAFE (survival activating factor enhancement)
- 14 RVP (rapid ventricular pacing)
- 15 VF (ventricular fibrillation)
- 16 VT (ventricular tachycardia)
- 17 S.E.M. (standard error of mean)

1 **Introduction**

2 Ischaemic heart diseases including acute myocardial infarction are the leading cause of
3 death in industrialized countries. Reperfusion therapy for infarction allows rapid return of
4 blood flow to the ischaemic myocardium and decreases mortality rate. However, early
5 reperfusion itself is accompanied by deleterious events: occurrence of life-threatening
6 arrhythmias, no-reflow phenomenon, myocardial stunning and additional cell death (Yellon *et al.*,
7 2007). This paradoxical reperfusion injury caused by the restoration of blood flow and
8 oxygen supply (Yamada *et al.*, 1990) leads to increased infarct size, impaired contractile
9 function, and electric vulnerability, largely compromising clinical outcomes.

10 Ischaemic postconditioning (IPost) has emerged in the last decade as a potential
11 therapeutic intervention for limiting reperfusion injury (Zhao *et al.*, 2003; Ovize *et al.*, 2010).
12 The procedure is based on application of brief cycles of ischaemia/reperfusion (I/R)
13 immediately after a prolonged ischaemia and it has been reported to reduce myocardial
14 damage in both animal studies and in human clinical trials (Ovize *et al.*, 2010). Nevertheless,
15 some studies have reported the ineffectiveness of IPost both in animals and in humans (Dow
16 *et al.*, 2007; Hahn *et al.*, 2013). A possible explanation for the controversial results could be
17 that the outcome of postconditioning may depend on several factors such as failure to achieve
18 complete reperfusion during application of brief I/R cycles, the duration of index ischaemia,
19 the algorithm of postconditioning manoeuvre, gender, age, and temperature (Skyschally *et al.*,
20 2009b). In addition, comorbidities like hyperlipidaemia (Kupai *et al.*, 2009) and diabetes
21 (Miki *et al.*, 2012) may interfere with the infarct size-limiting effect of postconditioning.
22 These confounding factors indicate the necessity to develop new alternative methods and
23 models to induce postconditioning.

24 Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*,
25 1989), and it was shown that induction of either slow- or rapid heart rate before ischaemia
26 limits myocardial injury (Tosaki *et al.*, 1988; Bernier *et al.*, 1989; Hearse *et al.*, 1999).
27 Moreover, we have shown previously that short periods of rapid ventricular pacing (RVP)
28 applied before an index ischaemia has anti-ischaemic effects (pacing-induced
29 preconditioning) (Ferdinandy *et al.*, 1997a; Ferdinandy *et al.*, 1997b; Ferdinandy *et al.*, 1998).
30 However, the effect of short periods of RVP performed at the early phase of reperfusion has
31 not been investigated so far.

32 The exact molecular mechanism of myocardial postconditioning is not entirely clear.
33 Increasing evidence suggests that enhanced formation of cardiac peroxynitrite is involved in
34 cardioprotection afforded by both pre- (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*,

2001) and postconditioning (Kupai *et al.*, 2009; Li *et al.*, 2013). Kupai *et al.* have reported first that IPost failed to decrease infarct size in the presence of a peroxynitrite decomposition catalyst, thereby suggesting essential triggering role of peroxynitrite in postconditioning-induced cardioprotection (Kupai *et al.*, 2009).

Therefore, here we aimed to investigate whether RVP applied after index ischaemia has any effect on markers of reperfusion injury and we studied the role of peroxynitrite in the mechanisms of postconditioning. Furthermore, we looked at activation of reperfusion injury salvage kinase (RISK) and survival activating factor enhancement (SAFE) pathways and haem oxygenase 1 (HO-1) as possible downstream targets of RVP-induced postconditioning.

Materials and methods

Male Wistar rats were used in our previous and present studies. The studies conform to the 'Guide for the care and use of laboratory animals' published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by local ethics committees. The animals were kept at 12/12-hour light/dark cycle and had free access to standard laboratory chow and drinking water.

Isolated heart preparation

Isolated heart preparation was done as described in our previous studies with slight modifications (Ferdinandy *et al.*, 1997a; Kocsis *et al.*, 2012; Varga *et al.*, 2014). Rats were anaesthetised with diethyl ether, an anaesthetic not known to interfere with cardioprotection, and were given 500 U·kg⁻¹ heparin intravenously. Hearts were then isolated and perfused according to Langendorff at 37 °C with Krebs-Henseleit buffer containing NaCl 118 mM, NaHCO₃ 25 mM, KCl 4.3 mM, CaCl₂ 1.5 mM, KH₂PO₄ 1.2 mM, MgSO₄ 1.2 mM, glucose 11 mM, gassed with 95% O₂ and 5% CO₂. Hydrostatic perfusion pressure was kept constant at 100 cmH₂O (9.8 kPa) throughout the experiments. Coronary flow was measured by collecting coronary effluent for a period of time and was expressed as mL·min⁻¹.

A 3-0 silk suture was placed around the left anterior descending coronary artery (LAD) close to its origin and the snare was tightened by applying a 100 g hanging weight to induce regional index ischaemia. For IPost brief no-flow global ischaemia was performed by turning off the perfusion cannula. The presence of ischaemia was verified by monitoring coronary flow. Rapid ventricular pacing (600 bpm; 10 Hz) was performed by an electric stimulator (Experimetria, Budapest, Hungary) with double threshold square, 1 V, 1 mA and 5-ms impulses conducted by electrodes attached directly to the surface of the right ventricle

close to the apex and to the aortic cannula as described previously (Ferdinandy *et al.*, 1997a; Ferdinandy *et al.*, 1997b; Ferdinandy *et al.*, 1998). Heart rates were monitored (Isosys, Experimetria Inc., Budapest, Hungary) by recording epicardial electrocardiogram (ECG) throughout the whole duration of perfusion.

Relationship of the duration of reperfusion-induced ventricular tachyarrhythmia and infarct size: a meta-analysis

Meta-analysis was performed on ECGs and infarct size data from our six previous studies done in our laboratory on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion [Figure 1A]. Reperfusion-induced arrhythmias were analysed in the first 10 min of reperfusion. Hearts presenting sustained (>10 min) tachyarrhythmia were excluded (n = 14). Three separate evaluations were done based on total duration of ventricular tachycardia (VT), ventricular fibrillation (VF), or VT+VF, respectively. Infarct size data were presented on the basis of duration (shorter or longer than 60 s) of VT, VF, or VT+VF. Infarct size data exceeding mean \pm two standard deviations were excluded from the analysis (n = 6).

Experimental design 1: testing the cardioprotective effect of rapid ventricular pacing

To examine whether RVP applied at the onset of reperfusion induces cardioprotection, isolated hearts were perfused as shown on Figure 2A. Three experimental groups were designed: (1) ischaemia/reperfusion control, (2) ischaemic postconditioning, (3) and rapid ventricular pacing groups (n = 12 in each group). The I/R control group was subjected to 15 min equilibration period, followed by 30 min regional index ischaemia and 120 min reperfusion. IPost was induced by six consecutive cycles of 10 s reperfusion and 10 s no-flow global ischaemia at the onset of reperfusion. In the RVP group the spontaneous rhythm of hearts was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles during the first 2 min of reperfusion.

To assess the severity of cellular damage in the myocardium, the activity of lactate dehydrogenase (LDH) enzyme from coronary effluents (collected during the first 5 min of reperfusion) was measured using a LDH-P kit (Diagnosticum, Budapest, Hungary) (n = 5 in each group). The enzyme activity ($\text{U} \cdot \text{mL}^{-1}$) measured in an effluent was multiplied with the corresponding coronary flow ($\text{mL} \cdot \text{min}^{-1}$) to give LDH release expressed as $\text{U} \cdot \text{min}^{-1}$.

To determine infarct size, the LAD was reoccluded at the end of reperfusion and hearts were stained with 0.1% Evans-blue to determine area at risk (Csonka *et al.*, 2010). Hearts were then frozen at -20°C and cut into approximately 2-mm thick slices. Each slice was

1 incubated at 37 °C for 10 min in 1% 2,3,4-triphenyl-tetrazolium-chloride solution dissolved in
2 phosphate buffer (pH 7.4). Slices were then fixed in 10% formaldehyde and scanned. Infarct
3 size was evaluated by planimetry (InfarctSize™ 2.4.b, Pharmahungary Group, Szeged,
4 Hungary) and normalised to area at risk.

5 To assess reperfusion-induced tachyarrhythmias (VT and VF), ECG was recorded
6 (Isosys, Experimetria Inc., Budapest, Hungary) during the entire perfusion protocol. Analysis
7 of arrhythmias was carried out according to the original Lambeth conventions (Walker *et al.*,
8 1988).

9 10 ***Experimental design 2: investigating the role of peroxynitrite and possible downstream*** 11 ***targets in rapid ventricular pacing-induced postconditioning***

12 To assess the possible role of peroxynitrite in cardioprotection induced by ischaemic- or
13 rapid ventricular pacing-induced postconditioning, in separate experiments, cardiac 3-
14 nitrotyrosine, a well-known peroxynitrite marker was determined. To confirm increased
15 peroxynitrite formation, cardiac superoxide anion was also measured. Furthermore,
16 involvement of molecular mechanisms (i.e. RISK and SAFE pathways, HO-1) that have been
17 implicated in cardioprotection (Hausenloy *et al.*, 2004; Lecour, 2009; Bak *et al.*, 2010) was
18 also investigated as possible downstream targets of RVP-induced postconditioning.

19 Hearts were subjected to 15 min equilibration period, followed by 30 min regional
20 ischaemia and 7 min reperfusion with or without IPost or RVP [Figure 4A]. At the end of
21 reperfusion myocardial samples were taken from the ischaemic zone of the left ventricle for
22 3-nitrotyrosine measurement and western blot analysis (n = 5 in each group). Sampling was
23 done by an oblique cut from the origin of the LAD toward the right side of the apical area that
24 involves the majority of the anterior wall of the left ventricle as well as the apex of the heart.
25 Samples were rapidly freeze-clamped, powdered with a pestle and mortar in liquid nitrogen,
26 and stored in cryovials at -80 °C until further analysis. Sampling for *in situ* detection of
27 superoxide anion was done in separate experiments (n = 3 in each group) using the same
28 perfusion protocol [Figure 4A]. Approximately 3-mm thick transverse slices were cut from
29 the middle of the ventricles, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek,
30 Zoeterwoude, Netherlands), carefully frozen in isopentane precooled in liquid nitrogen, and
31 stored at -80 °C until sectioning with a microtome.

32 Cardiac free 3-nitrotyrosine content, a marker of peroxynitrite, was measured by
33 enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA) according to
34 the manufacturer's instructions (Kupai *et al.*, 2009; Kocsis *et al.*, 2012). Briefly, homogenates

1 were incubated overnight with nitrotyrosine acetylcholinesterase tracer and anti-nitrotyrosine
2 rabbit IgG in microplates precoated with mouse anti-rabbit IgG. Ellman's reagent was used
3 for development. Free nitrotyrosine content was normalised to protein content of cardiac
4 homogenate and expressed as ng per mg protein.

5 Superoxide anion (O_2^-) is a reactive oxygen radical that reacts with nitric oxide to form
6 peroxynitrite. The *in situ* fluorescent dihydroethidium staining was performed to evaluate
7 intracellular production of superoxide anion (Varga *et al.*, 2013). Unfixed frozen heart
8 sections (30 μ m) were placed on glass slides and incubated in 10^{-6} mol·L⁻¹ dihydroethidium
9 (Sigma, St. Louis, MO, USA) in PBS buffer (pH 7.4) at 37 °C for 30 min in a dark humidified
10 container. Fluorescence was then detected by a fluorescent microscope (Nikon, Japan) with a
11 590 nm long-pass filter. Images of the hearts were collected digitally (n = 20 in each heart),
12 integrated density were evaluated by ImageJ 1.44p software and expressed in arbitrary unit.

13 The involvement of possible downstream targets in the mechanism of RVP-induced
14 postconditioning was examined by standard Western blot techniques (Kocsis *et al.*, 2008;
15 Fekete *et al.*, 2013). Tissue samples were homogenized with an ultrasonicator (UP100H
16 Hielscher, Teltow, Germany) in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5%
17 sodium deoxycholate, 5 mM EDTA, 0.1% SDS, 1% NP-40) supplemented with protease
18 inhibitor cocktail (Sigma, St. Louis, MO, USA), PMSF, NaF and Na₃VO₄. The crude
19 homogenates were centrifuged at 10,000 x g for 10 min at 4 °C. After quantification of
20 protein concentrations of the supernatants using BCA Protein Assay Kit (Pierce, Rockford,
21 IL, USA), 20 μ g (50 μ g for HO-1) reduced and denaturated protein was loaded and SDS-
22 PAGE (10% gel, 90 V, 1.5 h) was performed followed by transfer of proteins onto
23 nitrocellulose membrane (20% methanol, 35 V, 2 h). Membranes were blocked for 1 h in 5%
24 w/v bovine serum albumin (BSA) at room temperature and then incubated with primary
25 antibodies against phospho(Ser473)-Akt 1:500, Akt 1:2000, phospho(Thr202/Tyr204)-
26 Erk1/Erk2 1:2000, Erk1/Erk2 1:1000, phospho(Tyr705)-Stat3 1:2000, Stat3 1:2000 (Cell
27 Signaling, Beverly, MA, USA; overnight, 4 °C, 5% BSA) or HO-1 1:2000 (Enzo Life
28 Sciences, Plymouth Meeting, PA, USA; 2 h, room temperature, 1% milk) or GAPDH
29 1:10,000 (Cell Signaling, Beverly, MA, USA; 1 h, room temperature, 1% milk). After
30 incubation with HRP-conjugated secondary antibody 1:5000 (1:20,000 for GAPDH) (Dako
31 Corporation, Santa Barbara, CA, USA; 1 h, room temperature, 1% milk), membranes were
32 developed using enhanced chemiluminescence kit (Pierce, Rockford, IL, USA).

33 To further prove that both IPost and RVP protocols (i.e. application of brief
34 ischaemia/reperfusion or rapid ventricular pacing) facilitate peroxynitrite formation, 3-

1 nitrotyrosine was measured in the absence of index ischaemia. Effect of the protocols on
2 possible downstream targets of peroxynitrite (i.e. RISK and SAFE pathways) was also
3 examined in the absence of preceding index ischaemia.

4 In this set of experiments, the time course of perfusion protocol was adjusted to the
5 previous setup without index ischaemia [Figure 5A]. In the normoxic perfusion group (n = 8)
6 hearts were perfused for 52 min. In the repeated brief I/R group (n = 7) hearts were subjected
7 to 45 min perfusion followed by 6 x 10/10-s cycles of no-flow global I/R and 5 min
8 reperfusion. In the repeated brief RVP group (n = 8), the spontaneous rhythm of the hearts
9 was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles after 45 min
10 perfusion. At the end of perfusion, cardiac free 3-nitrotyrosine level was determined and
11 RISK as well as SAFE pathways were examined as described above.

13 ***Statistical analysis***

14 Data were expressed as mean \pm S.E.M and analysed with unpaired t-test, one-way
15 analysis of variance (ANOVA), or Fisher's exact test as appropriate. If a difference was
16 established in ANOVA, Fisher's Least Significant Difference (LSD) post hoc test was applied.
17 Differences were considered significant at $p < 0.05$.

19 **Results**

20 ***Duration of reperfusion-induced ventricular tachycardia and/or fibrillation is associated*** 21 ***with decreased infarct size***

22 Meta-analysis of six separate studies previously performed in our laboratory using the
23 same experimental protocol (i.e. isolated rat hearts subjected to I/R) showed that the presence
24 of VT, VF, or VT+VF with a total duration of longer than 60 s in the first 10 min of
25 reperfusion was associated with a markedly decreased infarct size [Figure 1B], respectively.
26 In this analysis a larger area at risk was associated with longer than 60 s total duration of
27 VT+VF [Figure 1C].

29 ***Rapid ventricular pacing exerts cardioprotective effect: limits the infarction and*** 30 ***reperfusion-induced arrhythmias***

31 In order to assess the possible cardioprotective effect of RVP, the extent of myocardial
32 infarction (LDH release and infarct size) was measured and reperfusion-induced arrhythmias
33 were analysed.

1 The post-ischaemic LDH release was significantly reduced by RVP [Figure 2B]. IPost
2 also reduced LDH release, however, the difference did not reach the level of statistical
3 significance [Figure 2B]. Infarct size was significantly decreased by both IPost and RVP
4 [Figure 2C]. There was no difference in the area at risk of either experimental group
5 [Figure 2D].

6 The incidence of VT and VF was not affected significantly by IPost in our present study
7 [Figure 3]. In contrast, short periods of RVP decreased the incidence of reperfusion-induced
8 VT without having a significant effect on VF [Figure 3].

9 There was no difference in animal weight, heart wet weight, baseline heart rate,
10 coronary flow (baseline, beginning of ischaemia, end of reperfusion) between the
11 experimental groups [Table 1]. In contrast to IPost, coronary flow at the onset of reperfusion
12 was not changed by short periods of RVP compared to I/R control [Table 1].
13

14 ***Peroxynitrite is likely involved in rapid ventricular pacing induced-postconditioning***

15 To obtain some mechanistic insight into the beneficial effect of RVP, cardiac 3-
16 nitrotyrosine and superoxide were measured at the 7th min of reperfusion following the 30 min
17 index ischaemia.

18 Postconditioning induced either by IPost or by RVP significantly increased free
19 cardiac 3-nitrotyrosine level (a marker of peroxynitrite formation) [Figure 4B]. Moreover, the
20 peroxynitrite precursor superoxide anion was mildly, but significantly elevated in both
21 postconditioning groups [Figure 4C].

22 To further prove that the postconditioning manoeuvres induce nitrative stress, cardiac
23 3-nitrotyrosine was measured after the postconditioning stimuli applied following normoxic
24 perfusion without index ischaemia. The application of brief I/R cycles or periodic RVP
25 increased the cardiac formation of 3-nitrotyrosine in the absence of index ischaemia
26 [Figure 5B].
27

28 ***Downstream mechanisms of rapid ventricular pacing-induced cardioprotection differs from*** 29 ***that of ischaemic postconditioning***

30 To elucidate the possible downstream targets of RVP, RISK and SAFE pathways as
31 well as HO-1 were investigated either in the presence or absence of index ischaemia.

32 Both postconditioning methods non-significantly enhanced Akt phosphorylation after
33 index ischaemia at the beginning of reperfusion without affecting phosphorylation of Erk1/2
34 and Stat3 [Figure 4E, F]. Protein level of HO-1 was increased by IPost but not RVP

[Figure 4E, F]. In the absence of index ischaemia, applying short periods of RVP protocol increased Stat3 phosphorylation, in contrast to brief cycles of I/R [Figure 5C, D]. Phosphorylation of Akt and Erk 1/2 was not affected significantly by any of the interventions in the absence of index ischaemia [Figure 5C, D].

Discussion and conclusion

In our present study, using an isolated perfused rat heart model, we confirmed that IPost beneficially affects I/R injury. Moreover, we demonstrated for the first time in the literature that applying short periods of RVP at the onset of reperfusion also exerts cardioprotective effect as it attenuates reperfusion injury by decreasing infarct size and reperfusion-induced arrhythmias. We showed that RVP increased peroxynitrite formation either in the presence or absence of index ischaemia in a similar way to IPost. These findings suggest that the formation of peroxynitrite in early reperfusion is a key event in the development of cardioprotection elicited by IPost or RVP. However, we also demonstrated that the downstream mechanisms of RVP-induced cardioprotection and IPost seem to be partially different.

In a meta-analysis of our previous studies on isolated hearts subjected to I/R we analysed if there is an association between the duration of reperfusion-induced ventricular tachyarrhythmias (VT, VF, or VT+VF) and infarct size. It is well accepted in the literature that I/R induces cellular damage that makes the myocardium more susceptible to arrhythmogenesis, and thus reperfusion-induced arrhythmias are considered as indicators of I/R injury (Engelen *et al.*, 2003; Majidi *et al.*, 2009). For instance, Majidi *et al.* have reported that presence of reperfusion arrhythmia bursts in STEMI patients are associated with worse outcome (larger infarct size and decreased ejection fraction) (Majidi *et al.*, 2009). However, here we found surprisingly that longer than 60 s reperfusion-induced ventricular tachycardia/fibrillation was associated with decreased infarct size. In this analysis a larger area at risk was associated with longer total duration of VT+VF in accordance with literature data (Curtis *et al.*, 1989). Interpretation of these results is difficult since causality was not examined in these studies. A possible explanation for the results of our meta-analysis is that the size of infarction affects the occurrence of sustained VT and/or VF, while another possibility is that longer tachyarrhythmias at the beginning of reperfusion somehow attenuate infarct development. To the best of our knowledge, this latter approach has not been investigated in the literature, and therefore these findings served as a basis for our current

experimental study to investigate if exogenous application of controlled tachycardia induced by RVP at the onset of reperfusion is able to elicit cardioprotection.

Heart rate is known to play a role in the development of I/R injury (Bernier *et al.*, 1989) and its controlled modification may elicit cardioprotection. For instance, pharmacologically-induced bradycardia (Tosaki *et al.*, 1987), slow- (Tosaki *et al.*, 1988) or rapid (Ferdinandy *et al.*, 1998; Hearse *et al.*, 1999) pacing before ischaemia was reported to limit myocardial injury. Since the presence of longer reperfusion-induced tachyarrhythmias was associated with lower infarct size in our meta-analysis, we wanted to test whether exogenous rapid pacing exerts protection. To the best of our knowledge, we demonstrated for the first time in the literature that the application of short periods of rapid (600 bpm) ventricular pacing at the beginning of reperfusion reduces infarct size and reperfusion-induced arrhythmias.

In the present study, both RVP and classic IPost decreased infarct size. The beneficial effect of RVP on infarct size was further confirmed by a reduction of LDH release into coronary effluent. Infarct size is a key determinant of major clinical outcomes (mortality and morbidity of consequent heart failure) (Gibbons *et al.*, 2004), therefore, development of procedures which effectively decrease infarct size along with reperfusion therapy is in the focus of preclinical and clinical studies (Ovize *et al.*, 2010). IPost is a widely studied approach, and the infarct size reducing effect of this procedure was confirmed in various mice, rat, rabbit, dog, and swine animal models (Skyschally *et al.*, 2009b) as well as in clinical trials (Ovize *et al.*, 2010). However, some studies reported the ineffectiveness of IPost in animal models (Dow *et al.*, 2007; Skyschally *et al.*, 2009b) and in clinical trials (Hahn *et al.*, 2013). A possible explanation for the controversial results could be that the cardioprotective effect of IPost depends on several factors such as for instance (1) species, strain, gender, age of research animal; (2) experimental model and set up; (3) the duration of index ischaemia before reperfusion; (4) number and duration of brief I/R cycles; (5) technical difficulty to achieve complete reperfusion; (6) temperature; (7) presence of comorbidities. These confounding factors indicate the necessity to develop alternative methods of IPost and we suggest that RVP-induced postconditioning is a simple method that eliminates technical problems associated with induction of IPost.

Besides infarct size reduction, RVP-induced postconditioning decreased reperfusion-induced ventricular arrhythmias as well. Reperfusion therapy is accompanied by occurrence of arrhythmias (Krumholz *et al.*, 1991). Some of them are benign (e.g. accelerated idioventricular rhythm, the most common type) but other ones are potentially life-threatening malignant arrhythmias such as VT or VF that need to be managed in the clinical practice to

1 avoid fatal consequences. Based on literature data (Kloner *et al.*, 2006), IPost effectively
2 decreases ventricular arrhythmias. However, in our present study, solely RVP-induced
3 postconditioning reduced the incidence of reperfusion-induced VT with no significant effect
4 on VF. The reason for the inability of RVP to improve post-ischaemic VF is not clear.
5 However, one may speculate that some interacting triggers of reperfusion-induced VF (e.g.
6 reactive oxygen intermediates and calcium) may interfere with the possible anti-VF effect of
7 RVP (Hearse *et al.*, 1988).

8 Here we demonstrated that IPost and RVP-induced postconditioning enhanced
9 peroxynitrite formation at the onset of reperfusion after an index ischaemia. In addition,
10 postconditioning manoeuvres themselves (i.e. brief ischaemia/reperfusion and rapid
11 ventricular pacing) increased peroxynitrite formation in the absence of the index ischaemia.
12 Since peroxynitrite is reported as a possible trigger of IPost (Kupai *et al.*, 2009), based on our
13 current results, we propose that the enhanced peroxynitrite formation also plays a role in
14 triggering RVP-induced postconditioning. Back in 1997, Yasmin *et al.* reported that the level
15 of peroxynitrite increases during reperfusion, which contributes to reperfusion injury in
16 isolated rat hearts (Yasmin *et al.*, 1997). Further studies also confirmed that enhanced
17 peroxynitrite formation plays a central role in numerous cardiovascular diseases by inducing
18 oxidative, nitrative- and nitrosative stress (Pacher *et al.*, 2007). However, peroxynitrite was
19 demonstrated to have physiological functions (Lefer *et al.*, 1997) and to play a role in
20 triggering ischaemic preconditioning (Altug *et al.*, 2000; Altup *et al.*, 2001; Csonka *et al.*,
21 2001). We have previously reported for the first time that peroxynitrite is a trigger of IPost,
22 since the peroxynitrite scavenger, FeTPPS interfered with the cardioprotective effect of IPost
23 (Kupai *et al.*, 2009). Our results were confirmed by Li *et al.* showing that peroxynitrite is a
24 key mediator of IPost *in vivo* (Li *et al.*, 2013). Nevertheless, the possible mechanisms lying
25 downstream of peroxynitrite formation in postconditioning have not been elucidated.

26 Here we also looked at possible targets of endogenous peroxynitrite formation induced
27 by IPost or by RVP. Several studies have reported that the activation of RISK (Akt,
28 Erk1/Erk2) and SAFE (Stat3) pathways at the onset of reperfusion might play a role in the
29 cardioprotective effect of IPost (Hausenloy, 2009; Lecour, 2009). In other studies
30 overexpression of HO-1 was shown to reduce infarct size in the heart (Bak *et al.*, 2010) and
31 was implicated in pulmonary and hepatic IPost (Xia *et al.*, 2009; Zeng *et al.*, 2011). In our
32 present study, both IPost and RVP-induced postconditioning non-significantly enhanced Akt
33 phosphorylation without affecting Erk1/2 and Stat3 at the beginning of reperfusion. Although
34 several studies showed increased phosphorylation of Akt and/or Erk due to IPost (Tsang *et*

1 *al.*, 2004; Yang *et al.*, 2004), some recent papers suggested that postconditioning did not
2 activate RISK pathway in the early phase of reperfusion (Skyschally *et al.*, 2009a; Fekete *et*
3 *al.*, 2013). We also found here that IPost but not RVP increased HO-1 protein in the heart.
4 This effect of IPost on HO-1 is in agreement with findings of others in the lung and liver (Xia
5 *et al.*, 2009; Zeng *et al.*, 2011). We also examined the effect of postconditioning manoeuvres
6 (i.e. repeated brief cycles of ischaemia/reperfusion or rapid ventricular pacing) in the absence
7 of a preceding index ischaemia and found no activation of the RISK pathway. In these
8 experiments, Stat3 phosphorylation was increased only by short periods of RVP protocol.
9 Taken together, our present results indicate that (1) the downstream mechanisms of RVP-
10 induced cardioprotection and IPost are partially different, (2) HO-1 is likely not involved in
11 the cardioprotective effect of RVP-induced postconditioning, and (3) the precise role of the
12 RISK and SAFE pathways remains to be elucidated in future studies. Involvement of
13 alternative pathways in the protective effect of RVP-induced postconditioning is likely, and
14 may include for instance activation of NO-cGMP-PKG, sphingosine-, protein kinase C-, or
15 CGRP-mediated pathways (Heusch *et al.*, 2008; Bice *et al.*, 2014). Since endogenous NO-
16 cGMP play a role in protection against reperfusion injury by attenuating infarct size (Penna *et*
17 *al.*, 2006) and reperfusion-induced VF (Pabla *et al.*, 1995; Pabla *et al.*, 1996), investigation of
18 the exact role of NO in RVP would be interesting.

19 Although we clearly demonstrated that RVP induces cardioprotection when applied at
20 the onset of reperfusion, some further limitations of our study may be considered. First,
21 ventricular pacing was reported to have direct pro-arrhythmic effects caused by the stimulus
22 itself independently from the heart rate (Nakata *et al.*, 1990). Although in our study
23 ventricular pacing last only for short periods (6 x 10 s), and the incidence of reperfusion-
24 induced VF was not increased in the RVP group when compared to I/R controls,
25 consideration of pacing as an ectopic focus cannot be excluded. Second, in RVP-induced
26 postconditioning ventricles were activated in a non-physiological way in the present *ex vivo*
27 study. Although the atrio-ventricular conduction system of rats was reported to be suitable for
28 reaching 600 bpm heart rate by atrial pacing in an *in vivo* model (Gonzalez *et al.*, 1998),
29 further *in vivo* studies are needed to investigate the infarct size limiting effect of
30 postconditioning induced by rapid atrial or ventricular pacing at different rates. Third, our
31 study suggests that rapid heart rate at the early phase of reperfusion may contribute to
32 initiation of adaptive molecular mechanisms to prevent I/R-induced cellular damage.
33 However, further studies are needed to analyse (1) the precise molecular nature of these
34 mechanisms and (2) if reperfusion-induced spontaneous arrhythmias also trigger adaptive

1 mechanisms in the myocardium. Our findings may also suggest that reperfusion-induced
2 tachyarrhythmias require attention in future studies focusing on cardioprotection assessed by
3 infarct size.

4 In conclusion, application of short periods of rapid ventricular pacing at the onset of
5 reperfusion beneficially affects essential components of reperfusion injury: the infarct size
6 and reperfusion-induced ventricular arrhythmias. In addition, RVP increases peroxynitrite
7 formation, which likely plays a role in triggering cardioprotection similarly to IPost.
8 Nevertheless, downstream mechanisms in RVP-induced protection seem to be partially
9 different from that of IPost, and further research is needed to elucidate them. Since RVP
10 exerted a similar cardioprotective effect to IPost, we feel that RVP-induced postconditioning
11 may serve as an alternative experimental model of IPost. Moreover, RVP could be performed
12 in more controlled manner than applying brief I/R cycles in IPost, which is an important
13 technical advantage compared to IPost.

14 15 **Acknowledgements**

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22
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Table 1. Morphological and *ex vivo* haemodynamic parameters.

	I/R	IPost	RVP
Animal weight (g)	367 ± 8	358 ± 10	345 ± 10
Heart wet weight (g)	1.28 ± 0.03	1.22 ± 0.04	1.30 ± 0.06
Basal heart rate (bpm)	301 ± 11	291 ± 12	304 ± 8
Coronary flow (mL·min ⁻¹)			
Before ischaemia	18.8 ± 1.5	16.7 ± 1.2	18.7 ± 1.1
Beginning of ischaemia ^a	10.7 ± 1.0	9.0 ± 0.8	11.5 ± 1.0
Beginning of reperfusion ^b	16.5 ± 1.0	8.7 ± 0.6*	17.9 ± 0.7
End of reperfusion	11.5 ± 1.5	9.9 ± 0.9	11.8 ± 1.5

^a regional ischaemia

^b 6 x 10 s global ischaemia was applied to induce IPost in the first 2 min of reperfusion.

Coronary flow was measured by collecting coronary effluent for 2 min and then was expressed as mL·min⁻¹.

Results are expressed as mean ± S.E.M. **p* < 0.05 vs. I/R and RVP, one-way ANOVA.

I/R: ischaemia/reperfusion control, IPost: ischaemic postconditioning, RVP: rapid ventricular pacing

Figure legends

Figure 1. *Duration of reperfusion-induced ventricular tachycardia and/or fibrillation is associated with decreased infarct size: a meta-analysis.*

Flow chart of the meta-analysis (A) indicates that reperfusion-induced tachyarrhythmias and infarct size data from our previous studies on isolated rat hearts subjected to 30 min regional ischaemia and 120 min reperfusion were analysed in three separate ways considering the duration of either ventricular tachycardia (VT), ventricular fibrillation (VF) or both in the first 10 min of reperfusion. Results of the meta-analysis shows infarct size normalised to area at risk (B) and area at risk (C) in the presence of shorter (<60 s) or longer (>60 s) total durations of VT, VF, or VT+VF, respectively. Values are expressed as mean \pm S.E.M. * $p < 0.05$ vs. corresponding <60 s groups, unpaired t-test.

Figure 2. *Rapid ventricular pacing reduces post-ischaemic LDH release and infarct size.*

Experimental protocol (A), post-ischaemic LDH release (B), infarct size normalised to area at risk (C), area at risk (D). Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 120 min reperfusion. Ischaemic postconditioning was induced by 6x10-s/10-s cycles of reperfusion/no-flow global ischaemia. In the rapid ventricular pacing group, the autonomic rhythm of the hearts was replaced by 10-s pacing period (600 bpm; 10 Hz) in 6 alternating cycles at the onset of reperfusion. Coronary effluent was collected during the first 5 min of reperfusion for LDH activity determination (n = 5 in each group), the measured activities were multiplied by the corresponding coronary flow to give LDH release. Infarct size was measured at the end of reperfusion (n = 12 in each group). Values are expressed as mean \pm S.E.M. * $p < 0.05$ vs. I/R, one-way ANOVA.

Figure 3. *Rapid ventricular pacing attenuates reperfusion induced arrhythmias.*

Incidence of reperfusion-induced ventricular tachycardia (A) and fibrillation (B) are shown. * $p < 0.05$ vs. I/R, Fisher's exact test-. I/R: ischaemia/reperfusion control, IPost: ischaemic postconditioning, RVP: rapid ventricular pacing. VT = ventricular tachycardia, VF = ventricular fibrillation.

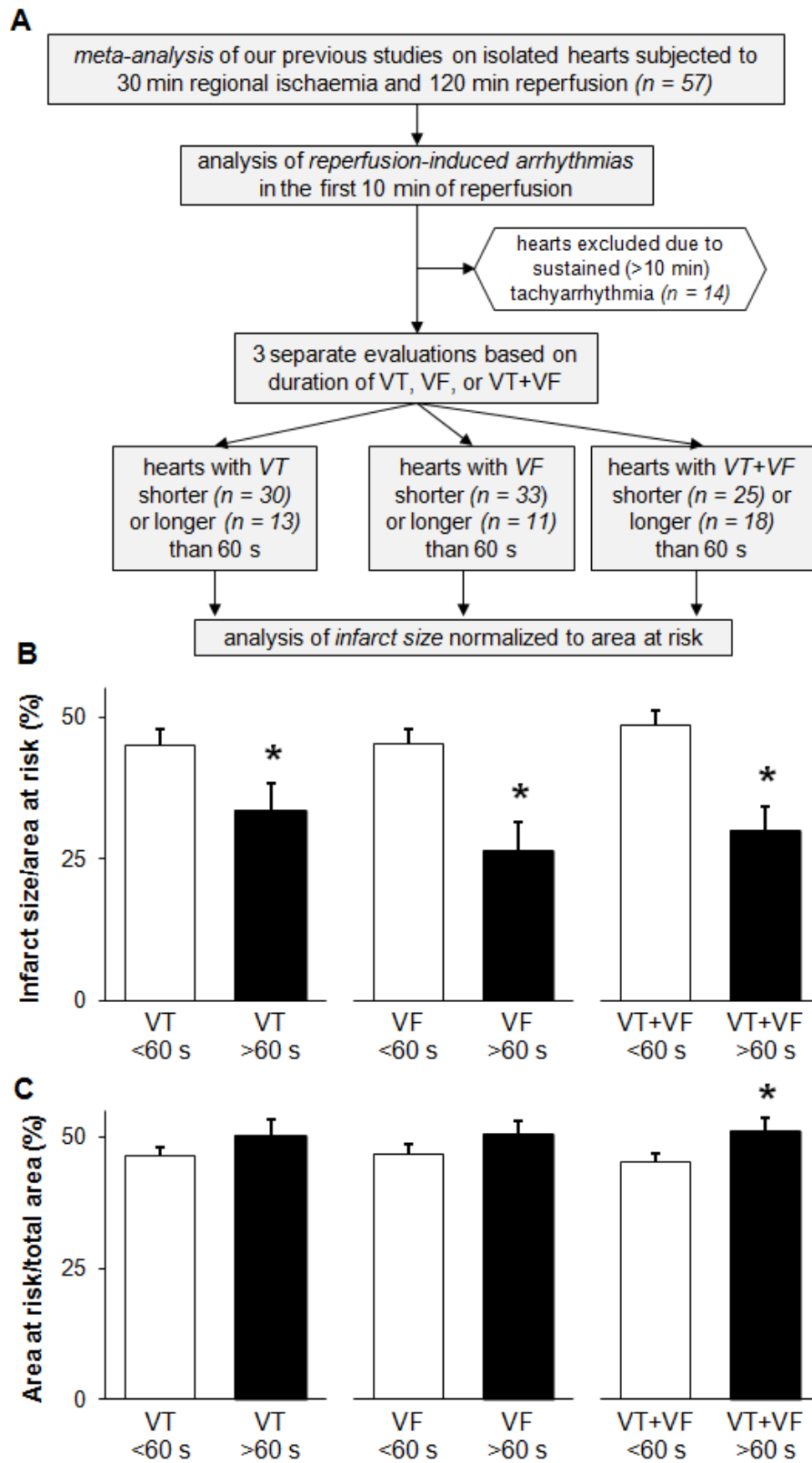
Figure 4. *Postconditioning by rapid ventricular pacing enhances formation of peroxynitrite and superoxide anion, effects on possible downstream targets.*

Experimental protocol (A), level of free cardiac 3-nitrotyrosine (B), representative images of *in situ* superoxide detection (C), quantification of *in situ* superoxide anion level (D), representative images (E), and quantification (F) of western blots of possible downstream targets. Hearts were subjected to 15 min equilibration period, followed by 30 min regional ischaemia and 7 min reperfusion with or without ischaemic postconditioning or rapid ventricular pacing. At the end of reperfusion myocardial samples were taken from the ischaemic zone of the left ventricle for biochemical analysis. The peroxynitrite marker, 3-nitrotyrosine was quantified by ELISA (n = 5 in each group). Transverse cardiac sections from three hearts per group were used for *in situ* detection of superoxide anion (n = 60 random images in each group). Activation of RISK (Akt, Erk1/2) and SAFE (Stat3) pathways as well as protein level of HO-1 was assessed by western blot. Values are expressed as mean \pm S.E.M. * $p < 0.05$ vs. I/R, one-way ANOVA. p-Akt: phospho(Ser473)-Akt, p-Erk1: phospho(Thr202)-Erk1, p-Erk2: phospho(Tyr204)-Erk2, p-Stat3: phospho(Tyr705)-Stat3, HO-1: haem oxygenase 1, GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Figure 5. *Postconditioning manoeuvres without a preceding index ischaemia enhances peroxynitrite formation, effects on possible downstream targets.*

Experimental protocol (A), level of free cardiac 3-nitrotyrosine (B), and representative images (C) and quantification (D) of western blots of possible downstream targets. After 45 min normoxic perfusion, repeated (6 x 10/10-s) brief cycles of no-flow global ischaemia/reperfusion (n = 7) or rapid ventricular pacing at 600 bpm/spontaneous rhythm of the hearts (n = 8) were applied followed by 5 min perfusion. In the normoxic perfusion control group (n = 8), hearts were perfused for 52 min. At the end of perfusion, cardiac free 3-nitrotyrosine level was determined by ELISA and activation of RISK and SAFE pathways were examined by western blot. Values are expressed as mean \pm S.E.M. * $p < 0.05$ vs. normoxic perfusion control, one-way ANOVA. I/R: ischaemia/reperfusion, RVP: rapid ventricular pacing. p-Akt: phospho(Ser473)-Akt, p-Erk1: phospho(Thr202)-Erk1, p-Erk2: phospho(Tyr204)-Erk2, p-Stat3: phospho(Tyr705)-Stat3, GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Figure 1.

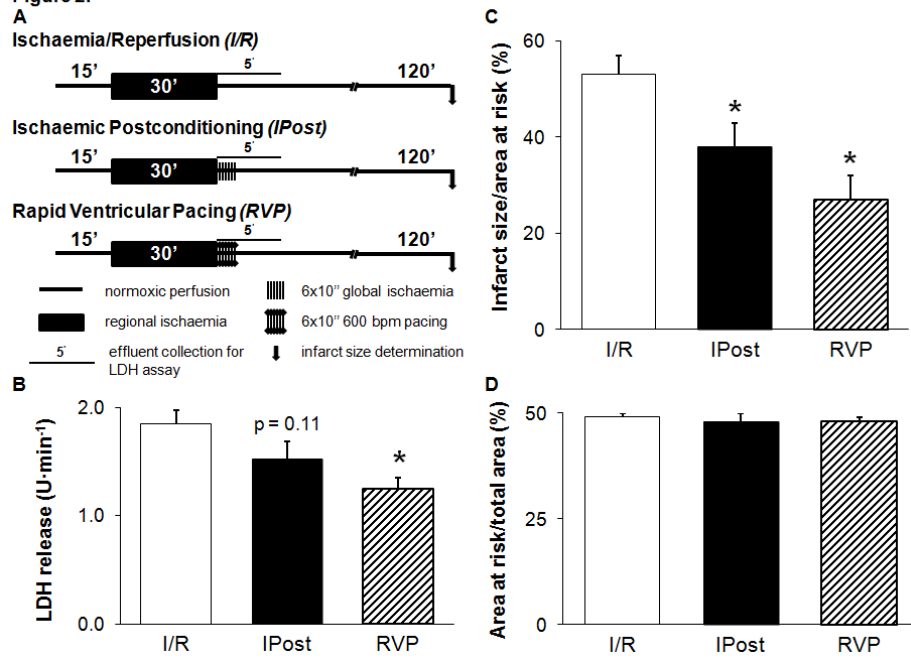


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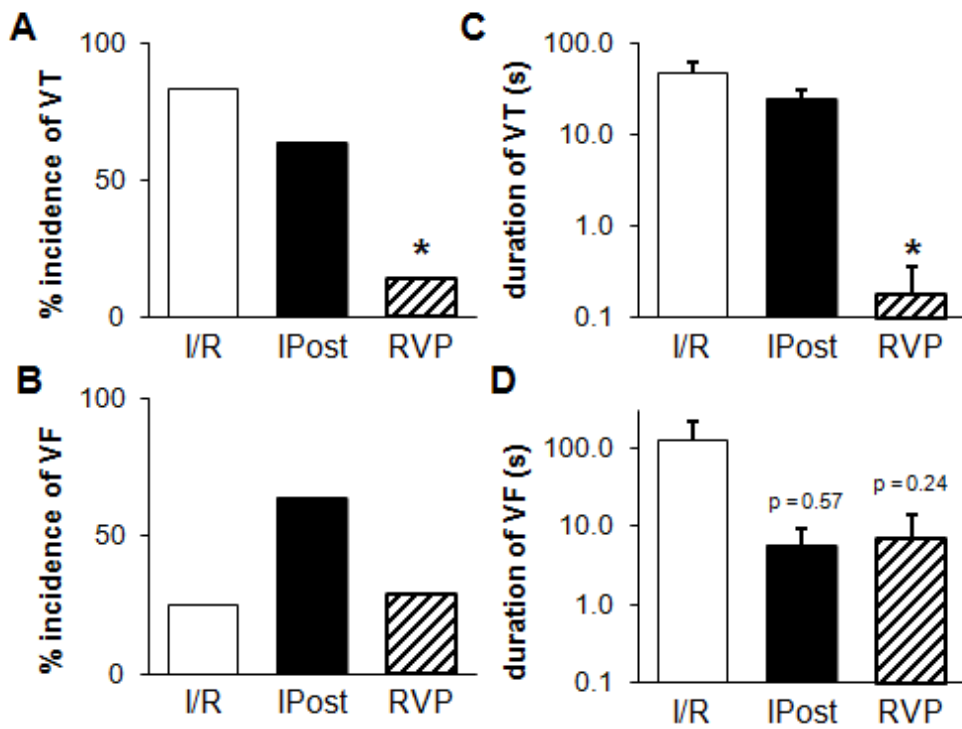
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Figure 2.



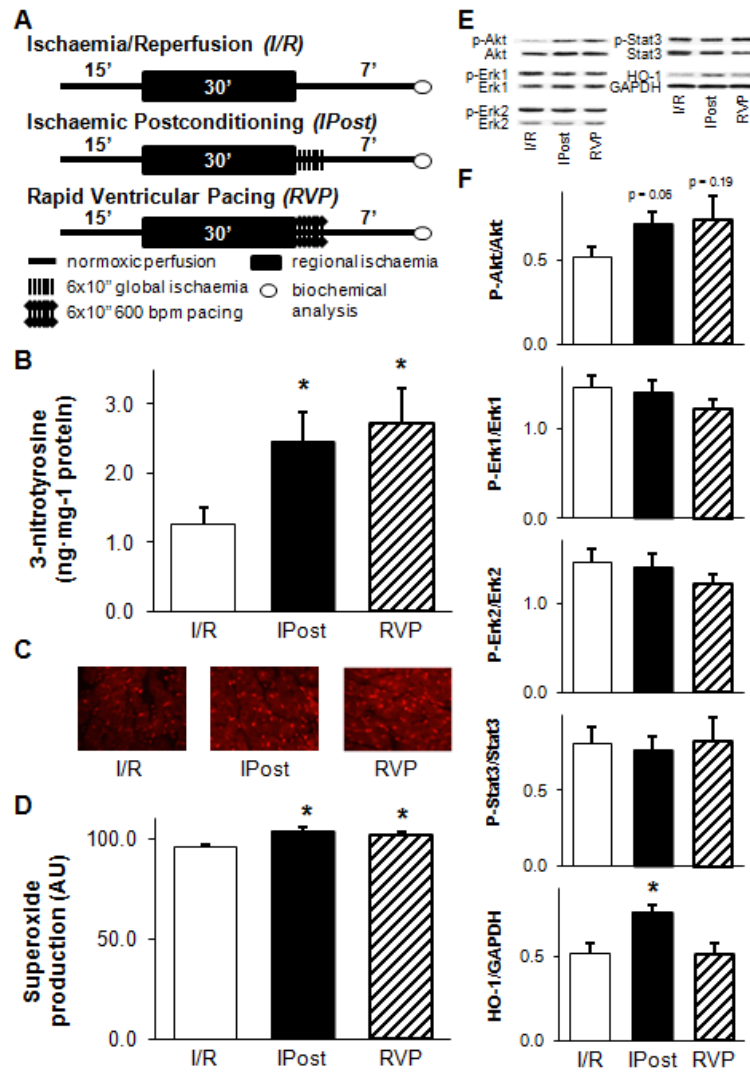
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Figure 3.



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Figure 4.



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Figure 5.

